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Early viral protein synthesis is necessary for NF- κ B activation in modified vaccinia Ankara (MVA)-infected 293 T fibroblast cells

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ARTICLE INFO

Article history:

Received 27 March 2009

Returned to author for revision 13 May 2009

Accepted 14 May 2009

Available online 18 June 2009

Keywords:

Vaccinia

NF- κ B

MVA

Vaccine

ABSTRACT

Modified vaccinia Ankara (MVA) is an attenuated vaccinia virus, and is a promising vaccine vector for variola and monkeypox viruses, as well as for other pathogens. The MVA determinants important for vaccine efficacy and immunogenicity are poorly defined. MVA infection of fibroblast cells activates NF- κ B, a characteristic not ascribed to wild-type vaccinia viruses. Thus, NF- κ B activation, and the subsequent upregulation of host immune molecules, could be one of the determinants for MVA's immunogenicity. We report that ERK2 phosphorylation, an event preceding and required for NF- κ B activation, occurred rapidly after virus infection. ERK2 and NF- κ B remained inert when virus endocytosis was prevented, suggesting that virus–host cell interactions were insufficient for activating NF- κ B. Inhibition of viral protein synthesis decreased NF- κ B activation, and elimination of intermediate and late gene expression did not alter MVA-induced NF- κ B activation. Thus, early gene expression activates NF- κ B.

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Introduction

Nuclear factor- κ B (NF- κ B) is a conserved eukaryotic transcription factor that is important for the immune system; it controls the expression of cytokines, MHC proteins, apoptotic factors, and cell adhesion molecules (Hoffmann, Natoli, and Ghosh, 2006). The canonical pathway triggering NF- κ B activation is well-characterized (Gilmore, 2006). Prior to activation, the p65/p50 heterodimeric NF- κ B complex is inactive in the cytoplasm, bound to its inhibitory protein, I κ B α . Diverse events, such as virus infection, will activate the I kappa kinase complex (IKK) complex. Once IKK phosphorylates I κ B α (DiDonato et al., 1997; Mercurio et al., 1997), I κ B α is degraded (Baldi et al., 1996; Chen et al., 1995). The NF- κ B NLS is then exposed and NF- κ B relocates to the nucleus, where the transcription factor binds to a conserved DNA sequence to regulate gene expression.

The control of NF- κ B activation during a natural virus infection can have great consequences (Hiscott et al., 2006). NF- κ B inhibition, for example, is detrimental for a host, by virtue of viral proteins dampening components of the innate and specific immune responses. In contrast, virus-induced NF- κ B activation may be beneficial to the host because anti-viral immune responses to neutralize virus infections are activated. The effect of the virus-mediated NF- κ B activation is well-documented for cells infected with vaccinia virus, a virus that is highly similar to variola virus and

an effective vaccine against smallpox (Moss, 2007). An attenuated vaccinia virus, such as the modified vaccinia Ankara (MVA) strain (Mayr, Hochstein-Mintzel, and Stickl, 1975), induces NF- κ B activation in host cells, whereas wild-type vaccinia strains do not (Oie and Pickup, 2001; Shisler and Jin, 2004).

Wild-type vaccinia viruses utilize myriad mechanisms to inhibit NF- κ B activation, including the synthesis of proteins that inhibit Toll-like-, interleukin-1 β receptor- and tumor necrosis factor receptor-induced signaling (Alcami et al., 1999; Alcami and Smith, 1992; Bowie et al., 2000). However, the molecular mechanism responsible for MVA-induced NF- κ B activation is unknown. The elucidation of this viral mechanism is important information when understanding how MVA regulates immune responses *in vivo*, and in considering the design of future MVA-based vaccines against smallpox or other infectious diseases. To this end, the stage of the MVA lifecycle responsible for activating NF- κ B was identified in the 293T fibroblast cell line. Using molecular and pharmacological inhibitors, it was found that virus binding to or entry into host cells was not sufficient to trigger the activation of this cellular transcription factor, nor did Toll-like receptor signaling appear to be important for the MVA phenotype. Instead, viral protein expression was required for the NF- κ B nuclear translocation in the 293T fibroblast cell line. Poxviral gene expression is temporal, and ORFs are grouped into early, intermediate or late genes, based on promoter sequences and time of expression during infection (Moss, 2007). MVA infections under conditions preventing intermediate or late gene expression did not ablate NF- κ B or ERK2 activation, suggesting that early gene expression is responsible for this phenotype.

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Results

NF- κ B and ERK2 activation occur early during MVA infection, and ERK2 phosphorylation precedes NF- κ B activation

It was shown previously that MVA infection of 293T cells results in host cell NF- κ B activation (Oie and Pickup, 2001; Shisler and Jin, 2004). To determine when this event occurred during poxvirus infection, we detected NF- κ B nuclear translocation, a hallmark of NF- κ B activation, in virus-infected cells by using EMSA. When NF- κ B is present in the nuclear extracts, and therefore active, it binds to radiolabeled oligonucleotides and retards their mobility. While there were multiple bands present in all lanes, one unique band was present in the lane containing extracts from infected cells harvested at 2–12 h post-infection (Fig. 1A). This same band increased in intensity until 4 h post-infection, remained relatively high until 10 h post-infection, and then decreased in intensity at 12 h post-infection (Fig. 1A). It was verified that the unique mobility-shifted band indeed contained the NF- κ B complex: when antibody recognizing the p65 subunit of NF- κ B was present, and presumably binding to NF- κ B, the unique band disappeared and a higher-mobility band appeared. The NF- κ B-containing band disappeared in reactions containing of non-labeled oligonucleotides possessing NF- κ B binding sites, but remained when non-labeled oligonucleotides instead possessing the AP1 binding site were present.

Activation of the ERK2 protein is necessary for MVA-induced NF- κ B nuclear translocation in 293T cells (Gedey et al., 2006). Therefore, ERK2 phosphorylation should temporally precede MVA-induced NF- κ B activation. To verify this, the presence of the active, phosphorylated (phospho-) ERK2 was detected at different times post-infection by using immunoblotting. Indeed, ERK2 activation occurred before NF- κ B activation; a 42 kDa phospho-ERK2 band was detected in cells harvested as early as 30 min post-infection (Fig. 1B). ERK2 activation, as measured by the intensity of the phospho-ERK2-containing band, peaked between 3 and 4 h post-infection, paralleling maximal NF- κ B nuclear translocation in MVA-infected cells. Finally, ERK2 phosphorylation waned 6 h post-infection, and was no longer detectable 8–12 h post-infection (Fig. 1B), even though NF- κ B was still present in the nucleus of virus-infected cells at these times (Fig. 1A). The anti-phospho-ERK2 antibody also cross-reacts with the highly similar phospho-ERK1 protein, as is evidenced by the presence of a higher molecular weight, 44 kDa band in most samples (Fig. 1B). The differences in phosphorylated ERK2 levels were not due to uneven protein loading since similar amounts of ERK1 and ERK2 proteins were detected in each reaction (Fig. 1B). The density of phospho-ERK2-containing bands was quantified by using densitometry. Values for phospho-ERK2-specific signals were computed by dividing the value of the signal obtained from lanes containing lysates from virus-infected cells by the value of the signal obtained from the lane containing lysates from mock-infected cells. The differences in phospho-ERK2 band intensities were represented graphically, and mimicked the trends observed in the immunoblot in Fig. 1B. When comparing the phospho-ERK2 levels, the density of the phospho-ERK2 band the lane containing lysates from mock-infected cells was set at a value of “1,” and the value for other treatments (virus infection) was presented as fold-increases over the value obtained for mock-infected cells. For this particular immunoblot, the phospho-ERK2 levels in cells at 3 h post-infection is approximately 9-fold higher than that observed during mock infection.

MVA does not utilize the MyD88 accessory protein to activate NF- κ B in 293T cells

MVA activation of dendritic cells (DCs) requires, in part, the Toll-like receptor 9 (TLR9), an intracellular molecule known to trigger NF- κ B activation (Samuelsson et al., 2008). Since NF- κ B activation is

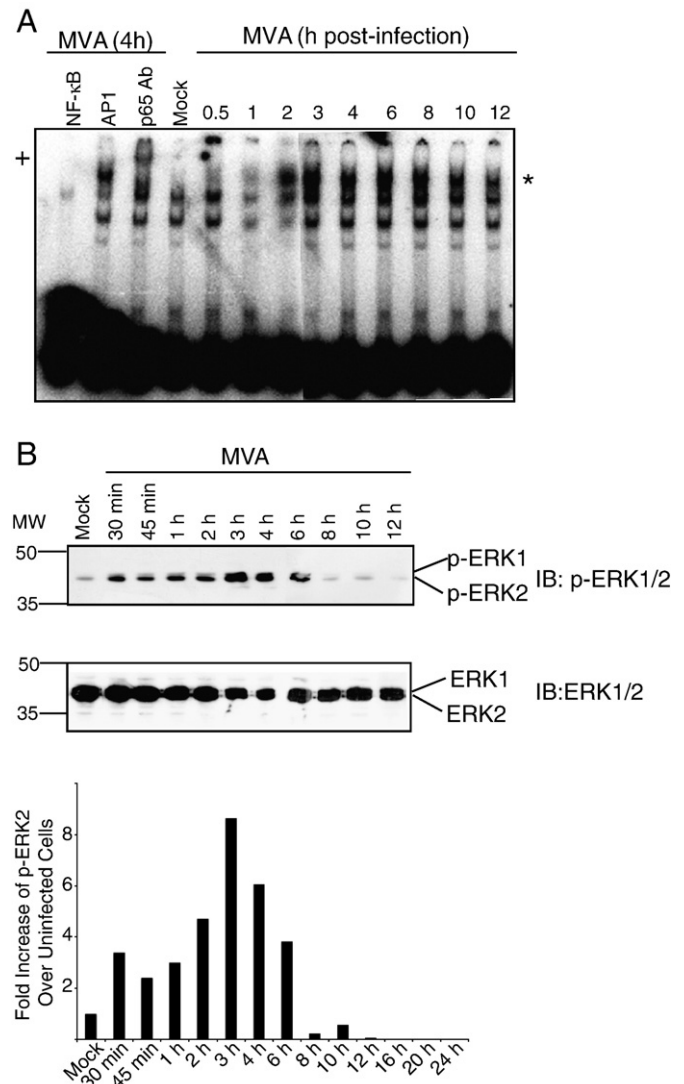


Fig. 1. Temporal NF- κ B nuclear translocation and ERK1/2 phosphorylation during vaccinia infection. 293T monolayers were either mock-infected or infected with MVA (MOI = 10). At the times indicated post-infection, cells were collected and lysed, and cytoplasmic and nuclear extracts were isolated. (A) 5 μ g of nuclear-extracted proteins from each sample was incubated with 32 P-radiolabeled oligonucleotides containing consensus NF- κ B binding sites, and analyzed via an electromobility shift assay. The location of the mobility-shifted band containing NF- κ B is indicated by an asterisk (*). For some reactions, the nuclear-extracted proteins from MVA-infected cells (4 h post-infection) were also incubated with excess amount of non-radiolabeled oligonucleotides containing either NF- κ B binding sites (NF- κ B) or lacking NF- κ B binding sites (AP1), or with anti-NF- κ B antibodies recognizing the p65 subunit of the NF- κ B dimer (p65 Ab). The super-shifted band containing anti-p65 is indicated by the “+” symbol. (B) 20 μ g of the cytoplasmically extracted proteins was electrophoretically separated via SDS-12% PAGE. Proteins were transferred to PVDF membranes, and immunoblots (IBs) were subsequently probed with antiserum recognizing the phosphorylated forms of ERK2 and ERK1 (p-ERK1/2) or the unmodified forms of ERK1 and ERK2 (ERK1/2). Molecular weight markers (MW) in kDa are indicated on the left-hand side of the immunoblot. Densitometry analysis was performed to quantify the phospho-ERK2 levels in the immunoblot. The signal from the lane containing lysates from mock-infected cells was set as a value equal to one. The amount of phospho-ERK2 in lanes containing lysates from MVA-infected cells was graphically represented as a fold-increase above that observed for mock-infected cells.

necessary for DC activation (Rowe et al., 2008), we queried if the TLR signal transduction pathway was responsible for MVA-induced NF- κ B activation in 293T cells. To this end, 293T cells ectopically over-expressing dominant negative mutant MyD88 proteins were infected with MVA, and NF- κ B activation was measured via a luciferase-based reporter assay, in which a luciferase gene is under

the transcriptional control of an NF- κ B promoter. Results are shown in Fig. 2. Luciferase activity levels were increased about 7-fold in lysates from MVA-versus mock-infected cells, inferring that MVA activated NF- κ B. As previously published (Gedey et al., 2006), the over-expression of a dominant negative ERK2 protein diminished luciferase activity. In contrast, luciferase activity levels in pMyD88DN-transfected cells were higher than those observed for either pERK2DN or untransfected cells, suggesting that MVA-induced NF- κ B activation did not require MyD88-dependent TLR activation. To confirm that the dominant negative MyD88 protein was expressed at levels that were biologically significant, a separate set of pMyD88-transfected cells were incubated with interleukin-1 β (IL-1 β), a cellular cytokine that utilizes the MyD88 accessory protein to activate NF- κ B. As would be expected, IL-1 β -mediated luciferase activity was greatly diminished in pMyD88DN-transfected cells versus pcDNA3.1-transfected cells. Notably, MVA-induced luciferase activity remained unaffected in pMyD88DN-transfected cells, inferring that the ERK2 activation pathway remained intact in these cells.

Virus binding to the cell membrane and viral endocytosis is not sufficient to activate NF- κ B

Since NF- κ B activation and ERK2 phosphorylation occurred relatively early during virus infection, one of the following events was responsible: virus binding to the host cell, virus entry into the cell, early viral protein expression, or DNA replication. To test if virus binding to the host cell was sufficient to activate NF- κ B, the virus adsorption stage of infection occurred at 4 °C and in the presence of

anti-L1R antibodies. Infections then proceeded for 4 h at 37 °C. This treatment allows virus binding to the host cell, but prevents entry (Ichihashi, Takahashi, and Oie, 1994; Ramsey-Ewing and Moss, 1998). Under these conditions, there was a dramatic decrease in MVA-mediated NF- κ B nuclear translocation as compared to MVA-infected cells incubated in medium lacking antibodies or in medium with antibodies not specific to vaccinia virus (Fig. 3A). As would be expected, NF- κ B remained inactive if non-specific antibodies were present during mock infection or during infection with WR, a wild-type strain of vaccinia virus that inhibits host cell NF- κ B activation (Shisler and Jin, 2004). Similar to Fig. 1A, the addition of anti-p65 antibodies to reactions containing extracts from MVA-infected cells super-shifted the NF- κ B-containing band, verifying the presence of NF- κ B in the observed band.

The effectiveness of anti-L1 antibody treatment to prevent virus entry was measured indirectly, by detecting viral protein synthesis in lysates from infected cells (Fig. 3B). When probing cytoplasmic extracts from WR- or MVA-infected cells with polyclonal antiserum raised against whole vaccinia virions, several unique bands (110 kDa, 35 kDa, 27 kDa, 15 kDa) were present, indicating that the polyclonal anti-vaccinia virus antiserum recognized multiple vaccinia virus proteins. In comparison, these bands' intensities were greatly decreased when assaying lysates from anti-L1, MVA-infected cells (Fig. 3B), inferring that the anti-L1 treatment indeed impeded virus entry into cells. Since actin levels were similar in all samples, the differences in viral protein levels were not due to unequal protein levels in each reaction.

It was reported recently that vaccinia virus penetration of the host cell occurs via a dynamin-dependent endocytosis pathway, an event inhibited by dynasore (Huang et al., 2008). As shown in Fig. 3C, when virus infections occurred in the presence of dynasore, NF- κ B no longer translocated to the nucleus, indicating that virus binding and endocytosis was not sufficient for virus-induced NF- κ B activation. Detection of viral E3 protein, an early vaccinia protein expressed only after viral endocytosis, was utilized as a mechanism to indirectly confirm the effectiveness of dynasore treatment (Fig. 3D). Indeed, the intensity of band representing the 20 kDa E3 product was dramatically decreased when infections proceeded in the presence versus the absence of dynasore.

It was expected that ERK2 would remain inactive when MVA infections occurred in the presence of dynasore. As predicted, there was a decrease in MVA-mediated ERK2 activation in the presence of dynasore, as measured by detection of phosphorylated ERK2 by immunoblotting (Fig. 3D). When comparing the intensity of the phospho-ERK2 bands in this immunoblot by using densitometry, it was observed that the phospho-ERK2 levels in cells treated with 40 μ M dynasore was similar (approximately 1), regardless of whether cells were infected or mock-infected. While there was a slight increase in the phospho-ERK2 levels in MVA-versus mock-infected cells in conditions in which cells were treated with 80 μ M dynasore (1 versus 0.5), levels were still lower than those observed for MVA infection (approximately 2) in the absence of drug. For this immunoblot, the fold-increase in phospho-ERK2 levels in MVA-infected cells was lower than that observed for Fig. 1, and this may be due to the presence of DMSO. Detection of similar amounts of ERK1 and ERK2 proteins in each sample negated the possibility that differences in phospho-ERK2 levels were due to decreased amounts of proteins in each sample.

Viral protein expression is necessary for MVA-induced NF- κ B activation

Cordycepin is a nucleoside adenosine derivative that prevents the translation of poxviral mRNA (Esteban and Metz, 1973). Thus, its presence during infection would assess the role of viral proteins synthesis on MVA-induced NF- κ B activation. Since NF- κ B remained inactive when MVA infection occurred in the presence of

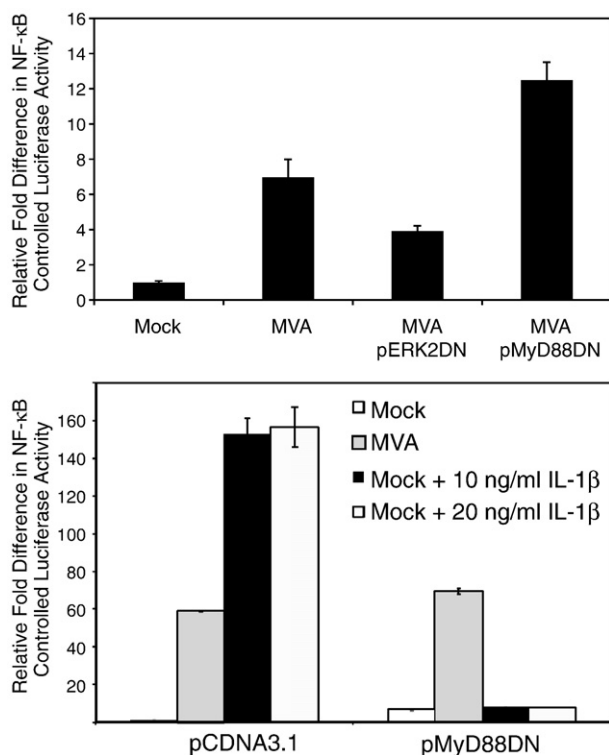


Fig. 2. MVA-induced NF- κ B activation in cells expressing a dominant negative mutant MyD88 protein. 293T cellular monolayers were co-transfected with pRL-null, pNF- κ B-luc and either pERK2DN or pMyD88DN, as indicated. At 24 h later, cellular monolayers were either mock-infected or infected with MVA (MOI of 10). An additional set of pMyD88DN-transfected cells was instead incubated with 10 ng or 20 ng IL-1 β per ml medium for 4 h. At 4 h post-infection cells were harvested, collected by centrifugation and incubated in passive lysis buffer. Firefly and sea pansy luciferase activities were measured, and the resultant ratios were normalized to that value obtained using untreated, uninfected cells.

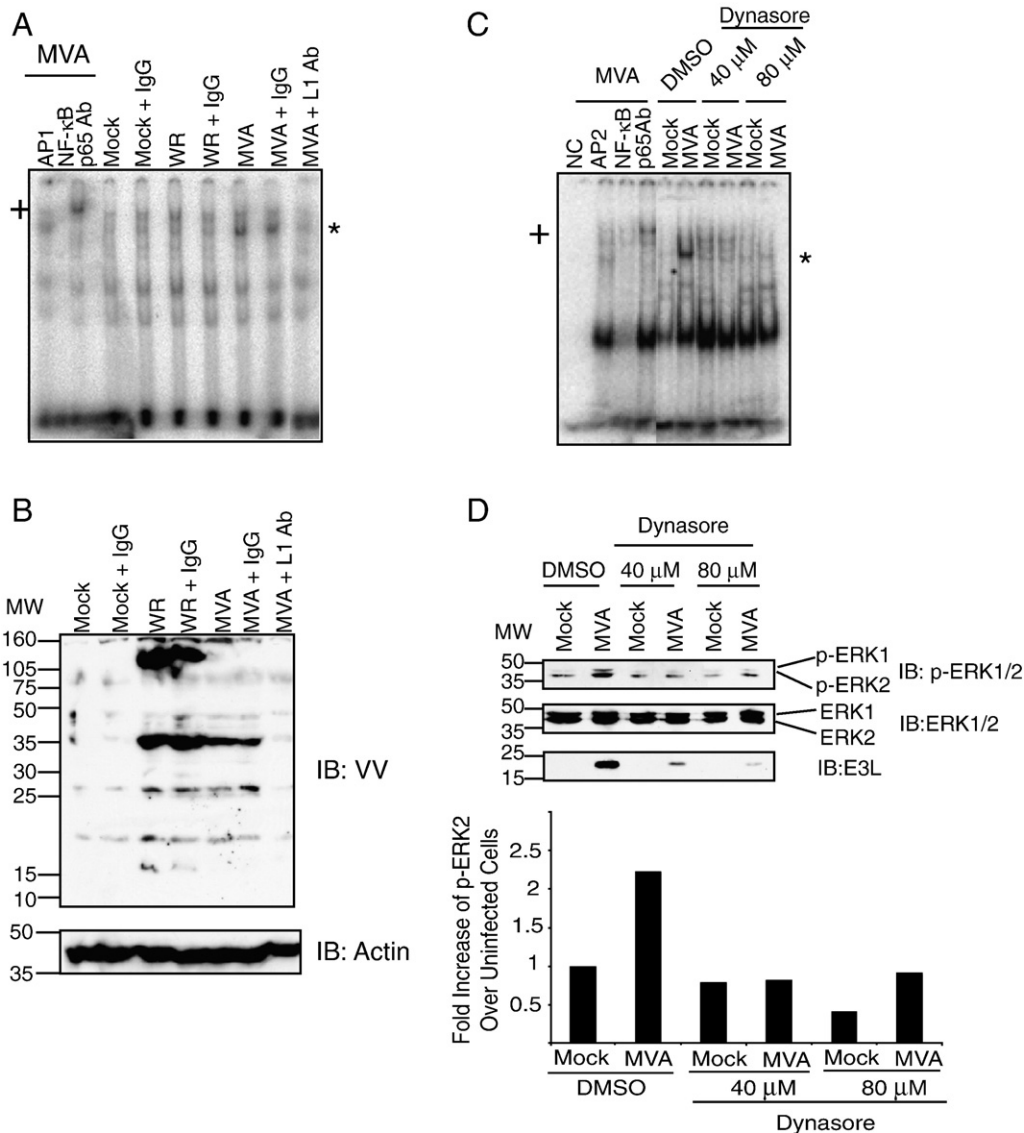


Fig. 3. The effect of inhibiting virus entry, but not attachment, on MVA-induced NF- κ B activation and ERK2 phosphorylation. (A and B) 293T monolayers were either mock-infected or infected with MVA or WR (MOI = 10). After a 1 h absorption period at 4 °C, medium was replaced with ice-cold medium containing either mouse IgG or anti-L1R antiserum (L1 Ab) for 15 min at 4 °C. Next, infections proceeded at 37 °C for 4 h. (C and D) 293T cells were incubated in medium either containing or lacking dynasore. At 30 min later, cells were incubated with MVA (MOI = 10) in medium either containing or lacking dynasore, and infections proceeded with the drug present for 4 h. For all figures, cells were harvested and lysed, and cytoplasmic and nuclear extracts were obtained as described in Fig. 1. (A and C) Nuclear-extracted proteins were incubated with 32 P-radiolabeled oligonucleotides containing consensus NF- κ B binding sites, and NF- κ B activation was analyzed by using an EMSA. The NF- κ B-containing band is indicated by an asterisk. For some reactions, nuclear extracts from MVA-infected cells were incubated with an excess amount of non-radiolabeled oligonucleotides or antiserum as described in Fig. 1. A reaction containing loading buffer only is labeled as "NC." A super-shifted band is denoted by "+." (B and D) Alternatively, cytoplasmically extracted proteins from each sample were analyzed by using immunoblotting. Membranes were subsequently probed with the indicated primary antibodies. Molecular weight markers (MW) are indicated on the left-hand side of the immunoblot. Densitometry analysis was performed to quantify the phospho-ERK2 levels in the immunoblot in panel D. The signal from the lane containing lysates from mock-infected cells was set as a value equal to one. The signal from lanes containing lysates from MVA-infected cells was graphically represented as a fold-increase above that observed for mock-infected cells. Values obtained from cells incubated in medium containing dynasore were also compared to mock-infected cells.

cordycepin (Fig. 4A), it was concluded that viral protein synthesis was necessary for NF- κ B activation. At the concentration used for this experiment, cordycepin was effective at inhibiting viral protein expression: immunoblotted viral proteins detected with anti-vaccinia or anti-E3 antiserum were decreased when infections occurred in the presence of cordycepin versus its absence (Fig. 4B). Actin levels in each of the cytoplasmic lysates were equivalent, inferring that the differences in observed viral protein levels were not due to differences in protein levels loaded in each sample.

To confirm the necessity of viral protein expression for MVA-induced NF- κ B activation, a separate experiment, using MVA

virions pre-exposed to ultraviolet (UV) light and psoralen, was performed. The use of these two treatments results in virions that still bind to and enter the host cell, yet a majority of the viral genome is not expressed or replicated (Tsung et al., 1996). In comparison to cells infected with untreated MVA, UV/psoralen-treated MVA no longer activated NF- κ B (Fig. 5A), confirming data in Fig. 4A. When luciferase reporter assays were used as an alternative method to indirectly measure NF- κ B activation, similar results were obtained. As shown in Fig. 5B, MVA infection of cells induced an increase in firefly luciferase activity approximately 7-fold greater than mock-infected cells, presumably reflecting NF- κ B activation. However, luciferase activity was decreased when cells

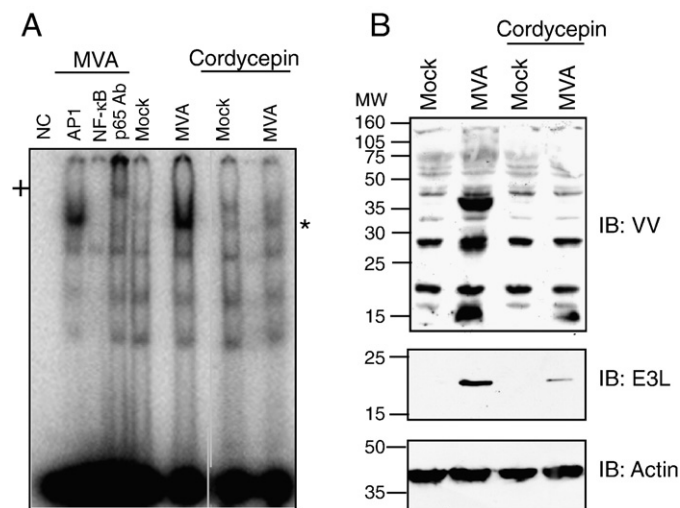


Fig. 4. The virus-mediated NF- κ B activation state in the presence of cordycepin. 293T cellular monolayers were incubated in medium either absent for or containing cordycepin (40 μ g/ml). At 30 min later, cells were either mock-infected or infected with MVA (MOI = 10). Where applicable, the drug was present in the medium during the adsorption stage of infection and for the duration of the infection. At 4 h post-infection, cells were lysed, and proteins from either the cytoplasm or the nucleus were isolated. (A) Nuclear-extracted proteins were analyzed for NF- κ B activity by EMSA, as described in Fig. 1. The location of mobility-shifted bands containing NF- κ B is indicated by an asterisk. For some reactions, nuclear extracts from MVA-infected cells were incubated with an excess amount of non-radiolabeled oligonucleotides or antiserum as described in Fig. 1. In one lane, only gel loading dye was added (NC). (B) Cytoplasmic proteins (20 μ g from each sample) were analyzed by using immunoblotting, as discussed in Fig. 1. IBs were probed with the indicated antibodies. Molecular weight markers (MW) are indicated on the left-hand side of the figure.

were instead infected with psoralen/UV-inactivated MVA virions (Fig. 5B), inferring that NF- κ B was inactive under these conditions.

Phospho-ERK2 was greatly decreased in cells infected with the treated virions as compared to those incubated with unmodified MVA (Fig. 5C), a result that was predicted. Equivalent levels of unmodified ERK2, and the highly similar ERK1, were detected in each sample, indicating that the differences observed for phospho-ERK2 levels were not due to differing protein levels present in each sample. The UV/psoralen treatment of MVA virions was effective for decreasing viral protein synthesis. As shown in Fig. 5C, when expressing phospho-ERK2 levels graphically, by using densitometry to measure phospho-ERK2, it was observed that phospho-ERK2 levels were 30-fold higher in MVA-infected cells versus mock-infected cells. In contrast, phospho-ERK2 levels were only 5-fold above mock-infected cells when MVA virions were pre-treated with UV and psoralen. UV/psoralen treatment of MVA dramatically decreased the synthesis of the early E3L protein, and the L1R late protein was no longer detected under these conditions, indicating that the UV/psoralen treatment of virions was sufficient to greatly decrease early gene expression.

Intermediate or late gene expression, but not early gene expression, is dispensable for MVA-induced activation of NF- κ B and ERK2

Poxvirus gene expression is temporal, with early genes expressed before viral DNA replication, and intermediate and late genes expressed after viral DNA replication (Moss, 2007). To determine if intermediate and late proteins were dispensable for MVA-induced NF- κ B activation, we infected 293T cells in the presence of cytosine arabinoside (Ara C), a drug that prevents viral DNA replication, and subsequent intermediate and late gene expression.

Under these conditions, the lack of intermediate and late protein expression did not affect the ability of MVA to activate host cell NF- κ B at 4 h post-infection, since the nuclear translocation of NF- κ B was still

detected in nuclear extracts (Fig. 5A). Furthermore, NF- κ B remained effective as a transcriptional activator of a luciferase reporter gene in MVA-infected cells, regardless of whether Ara C was present or absent during infection (Fig. 5B). Not surprisingly, virus-induced ERK2 phosphorylation was unaffected by the presence of Ara C during infection (Fig. 5C). The effectiveness of the drug in inhibiting intermediate and late protein expression is demonstrated in Fig. 5C, when immunoblotted lysates were probed for the presence of either an early product (the E3L product) or a late product (the L1R product). When comparing lysates from cells infected in Ara C-containing versus complete medium, there was a decrease in the amount of L1R product present, indicating a decrease in late protein synthesis. E3L protein expression was not affected by the presence of Ara C, as would be predicted.

As confirmation that late viral protein expression was dispensable for NF- κ B activation, 293T cells were instead infected with MVA Δ E3L, a mutant MVA construct deleted for the early E3L ORF. As reported by Ludwig et al. (2006), infection of 293T cells with MVA Δ E3L results in an abortive infection, in which candidate late proteins are not produced. Under these circumstances, NF- κ B remained active in MVA Δ E3L-infected cells, as detected by an EMSA (Fig. 6A). Importantly, the temporal regulation of NF- κ B during MVA Δ E3L infection was similar to that observed during infection with MVA, implying that the extended NF- κ B activation observed during MVA infection was solely due to early gene expression. Since ERK2 activation is required for NF- κ B activation (Gedey et al., 2006), it was not surprising to observe that phosphorylated ERK2 was detected in extracts from MVA Δ E3L-infected cells (Fig. 6B). As would be expected, the temporal activation of ERK2 was similar in MVA Δ E3L- and MVA-infected cells. The density of phospho-ERK2-containing bands was quantified by using densitometry. Values for phospho-ERK2-specific signals were computed by dividing the value of the signal obtained from lanes containing lysates from virus-infected cells by the value of the signal obtained from the lane containing lysates from mock-infected cells. In this experiment, phospho-ERK2 levels were highest at 1–3 h post-infection, with MVA infection inducing phospho-ERK2 levels 3–5-fold above mock-infected cells. While this activation profile was slightly different than that observed for Fig. 1, we still observed ERK2 activation early during infection, and then waning at times later in infection for both experiments. Phospho-ERK2 levels in MVA Δ E3L-infected cells were slightly lower than those of MVA-infected cells, inducing 2–4-fold above mock-infected cells. Regardless of virus used, phospho-ERK2 levels were similar to mock-infected cells by 4 h post-infection.

Discussion

Modified vaccinia virus Ankara infection, unlike wild-type vaccinia strains, activates the cellular NF- κ B transcription factor (Gedey et al., 2006; Oie and Pickup, 2001). The viral mechanism(s) responsible for this phenotype was heretofore unknown. Using several approaches, we identified that viral gene expression was required for this phenotype, with the expression of the early class of vaccinia ORFs correlating with MVA-induced NF- κ B activation. Two possibilities for this phenotype existed. First, the concomitant expression of all early proteins was activating NF- κ B via mechanisms such as the endoplasmic reticulum overload response (EOR) pathway. However, since MVA infection of 293T cells did not trigger calcium release (data not shown), an event occurring during EOR activation (Kuang et al., 2005), this possibility was unfavorable. An alternative hypothesis is that the expression of an individual early viral protein activates NF- κ B either directly or indirectly. Experimental approaches to test each of these theories are the subject of ongoing investigations.

Recent studies have investigated the effects of MVA infection on DC activation. Dendritic cells (DCs) are potent antigen presenting cells

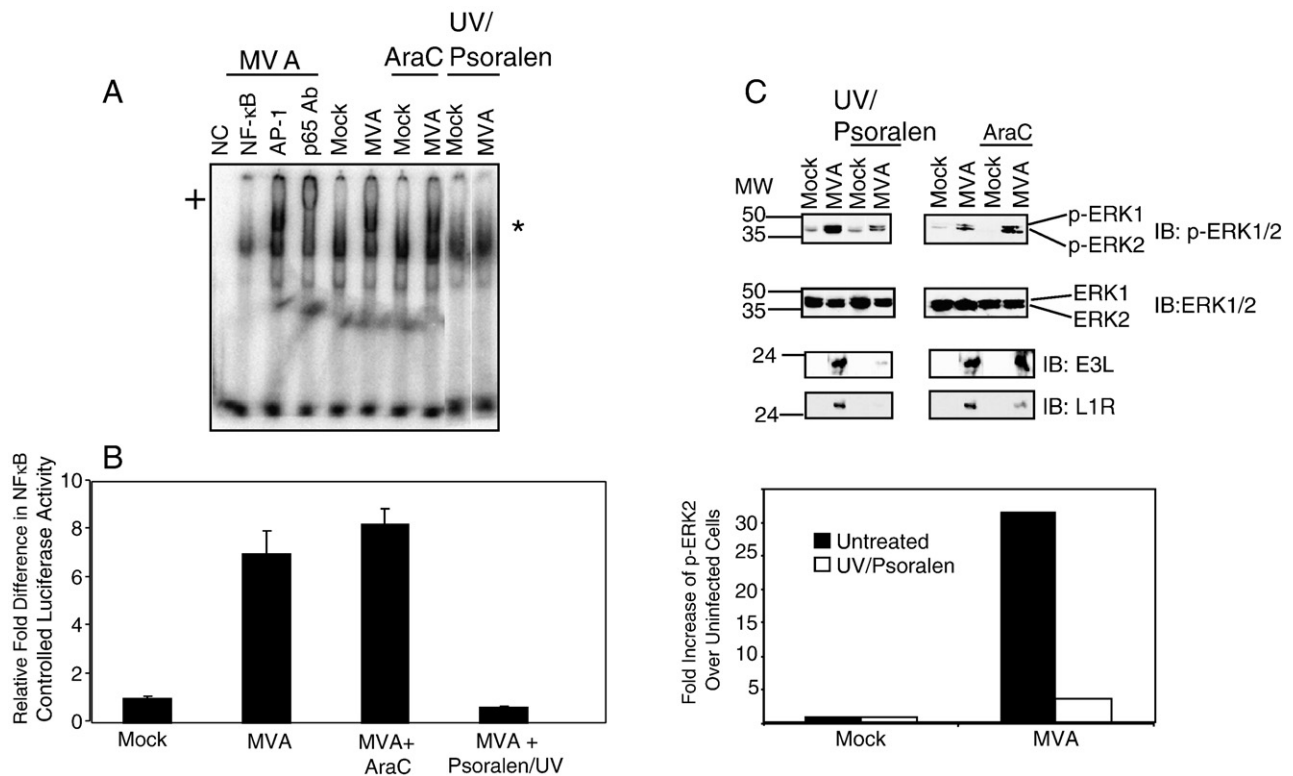


Fig. 5. The effect of either Ara C treatment of cells or psoralen/UV treatment of virions on MVA-induced NF- κ B activation and ERK2 phosphorylation. (A and C) Cellular monolayers were either mock-infected or infected with MVA (MOI of 10) in medium absent for or containing 40 μ g/ml Ara C. Alternatively, some MVA virions were incubated in PBS containing psoralen (10 μ g/ml) and subsequently treated with UV for 5 min before incubation with cellular monolayers (MOI=10). In all cases, at 4 h post-infection cells were harvested, collected by centrifugation and lysed, and cytoplasmic and nuclear proteins were isolated. For (A), nuclear-extracted proteins were analyzed for NF- κ B activation by EMSAs, as described in Fig. 1. The location of mobility-shifted bands containing NF- κ B is indicated by an asterisk. Some reactions contained nuclear extracts from MVA-infected cells and either anti-NF- κ B antibodies (p65 Ab), or excess amounts of non-radiolabeled oligonucleotides containing NF- κ B binding sites (NF- κ B) or lacking NF- κ B binding sites (AP1). A super-shifted band is indicated by a plus sign. A lane containing only loading buffer is labeled as NC. (B) Subconfluent 293T cell monolayers were transfected with pRL-null and pNF- κ Buc. 24 h later, cellular monolayers were either mock-infected or infected with MVA (MOI of 10) as described in (A). At 4 h post-infection cells were harvested, collected by centrifugation and incubated in passive lysis buffer. Firefly and sea pansy luciferase activities were measured, and the resultant ratios were normalized to that value obtained using untreated, uninfected cells. (C) Cytoplasmic extracts from either mock- or virus-infected cells were analyzed for cellular and viral proteins as described in Fig. 1. IBs were probed for either the phosphorylated (p-ERK1/2), or unphosphorylated forms of ERK1/2. Identical IBs were alternatively incubated with anti-E3L or anti-L1R antiserum. Molecular weight (MW) markers are on the left-hand side of the immunoblot. Densitometry analysis was performed to quantify the phospho-ERK2 levels in the immunoblot in panel C. The signal from the lane containing lysates from mock-infected cells was set as a value equal to one. The signal from lanes containing lysates from MVA-infected cells was graphically represented as a fold-increase above that observed for mock-infected cells. Values obtained from cells incubated with UV/psoralen-treated MVA were also compared to mock-infected cells.

to activate naive CD4⁺ and CD8⁺ T cells, making them important for the development of adaptive immune responses (Guermónprez et al., 2002). NF- κ B activation is necessary for DC activation (Rowe et al., 2008). One prediction from our results is that early protein synthesis is also required for MVA-induced DC activation. This model is in agreement with a report that UV-inactivation of MVA does not enhance virus-induced DC activation (Samuelsson et al., 2008). However, DC activation with UV-treated MVA has also been documented (Drillien, Spohner, and Hanau, 2004; Waibler et al., 2007), in contrast to our findings reported here. Additionally, MVA activation of DCs utilizes the TLR9, an intracellular molecule known to trigger NF- κ B activation (Samuelsson et al., 2008). However, the inhibition of TLR9-induced NF- κ B activation, via over-expression of a dominant negative MyD88 protein, did not decrease MVA-induced NF- κ B activation. Whether MVA possesses two different mechanisms for activating NF- κ B in fibroblasts (early protein synthesis required, TLR-independent) versus immune cells (early protein synthesis not required, TLR-dependent) is the subject of future studies. Yet another possibility is that UV irradiation of MVA is not as efficient for cross-linking the MVA genome as UV/psoralen treatment of MVA (Tsong et al., 1996). Thus, UV-irradiated MVA virions might allow for the transcription of viral ORFs, an event that would not occur with UV/psoralen-treated MVA virions. Finally, these differences could be due

to varying TLR protein expression levels in fibroblast versus immune cells.

ERK2 and NF- κ B activation occurred rapidly after virus infection, raising the possibility that proteins in the mature virion may activate NF- κ B when the virus is intracellular. However, we observed two experimental conditions in which the inhibition of viral protein synthesis rendered NF- κ B inactive in virus-infected cells (UV/psoralen treatment of virions and incubation of cells with cordycepin). These data support our position that nascent viral proteins are responsible for NF- κ B activation.

Curiously, NF- κ B remained active in MVA-infected cells for an extended period of time. Although MVA infection of human monocyte-derived DCs upregulates I κ B α gene expression (Guerra et al., 2007), it is likely that virus infection in 293T cells sequesters the auto-regulatory feedback loop for NF- κ B. Whether these patterns remain present in all fibroblasts versus immune cells, and whether this trait possesses physiological relevance, is unknown. Equally unlikely is a late gene product prolonging NF- κ B activation: cells infected with MVA Δ E3L, a deletion mutant virus that no longer expresses intermediate or late genes in 293T cells (Ludwig et al., 2005; Ludwig et al., 2006), have an NF- κ B activation profile that is identical to that of MVA-infected cells. A current favored model for this prolonged activation is that a viral product actively synthesized

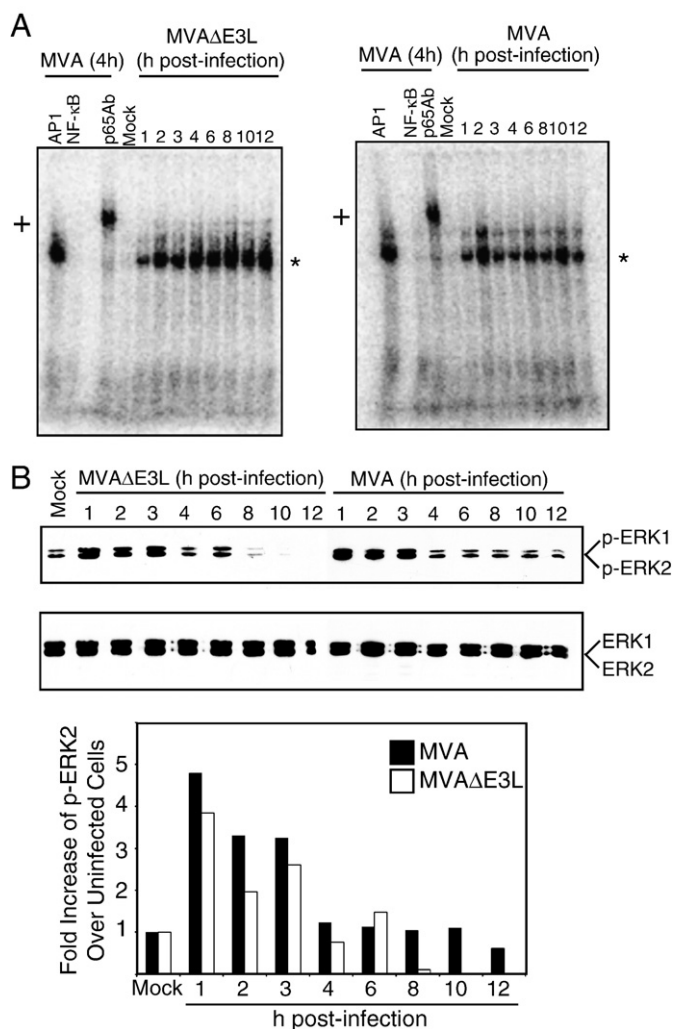


Fig. 6. The NF- κ B activation and ERK2 phosphorylation states in cells infected with MVA viruses either lacking or containing the E3L ORF. 293T cell monolayers were either mock-infected or infected with either MVA (MOI = 10) or MVA Δ E3L (MOI = 10). One hour after the absorption period, cells were incubated in fresh medium. Cellular monolayers were incubated at 37 °C for the indicated times. Cells were lysed, and cytoplasmic proteins and nuclear proteins were isolated. (A) Nuclear-extracted proteins were analyzed for NF- κ B nuclear translocation by using EMSAs as described in Fig. 1. The location of mobility-shifted bands containing NF- κ B is indicated by an asterisk. For some reactions, nuclear extracts from MVA-infected cells were incubated with an excess amount of non-radiolabeled oligonucleotides or antiserum as described in Fig. 1. A super-shifted band containing NF- κ B is indicated by a plus sign. In one lane, only gel loading dye was added (NC). (B) 20 μ g cytoplasmically extracted proteins from each sample were analyzed by immunoblotting. Membranes were probed with antibodies recognizing either the phospho- or unmodified forms of ERK1 and ERK2. Molecular weight (MW) markers are indicated. Densitometry analysis was performed to quantify the phospho-ERK2 levels in the immunoblot in panel B. The signal from the lane containing lysates from mock-infected cells was set as a value equal to one. The signal from lanes containing lysates from MVA- or MVA Δ E3L-infected cells was graphically represented as a fold-increase above that observed for mock-infected cells.

throughout infection induces this phenotype. Yet another possibility is that an early protein that is stable throughout infection can activate NF- κ B throughout infection. Data obtained using the MVA Δ E3L virus for infection cannot rule out either of the above possibilities.

Poxviruses are complex viruses that produce multiple immunoevasion products, with many of these products important for virulence (Moss, 2007). Data shown here indicate that vaccinia also expresses proteins to stimulate the immune response. Why the wild-type virus maintains multiple genes that encode proteins with opposing actions is an important conundrum, given the traditional idea that viruses are evolutionarily driven to minimize the number of genes they encode.

This finding may indicate that temporal regulation of NF- κ B activation is more important than overall inhibition of NF- κ B during vaccinia virus pathogenesis, and leads to a new appreciation for the regulation of this transcription factor during a poxvirus lifecycle. Notably other viruses, such as herpes viruses, also express proteins that either activate or inhibit NF- κ B (Hiscott et al., 2006), suggesting that control of this transcription factor by complex viruses is a common strategy for survival and pathogenesis.

Materials and methods

Cells and viruses

The human kidney fibroblast HEK293T (293T) and baby hamster kidney (BHK-21) cell lines were obtained from the American Type Culture Collection (ATCC). All cells were cultivated in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal calf serum (FCS; Hyclone), referred to as complete medium. MVA is an attenuated strain of vaccinia virus (Mayr, Hochstein-Mintzel, and Stickl, 1975). MVA Δ E3L, an MVA-based virus in which the E3L ORF has been deleted (Ludwig et al., 2005), was a gift from Dr. Gerd Sutter (Paul-Ehrlich Institut). MVA Δ E3L was amplified in BHK-21 cells, while MVA was amplified in primary CEF cells (Charles Rivers Laboratories). The Western Reserve (WR) strain of vaccinia virus, which was amplified in BHK-21 cells, is considered to be a wild-type strain of vaccinia virus.

Conditions for virus infections

Subconfluent 293T cell monolayers in 6-well plates were either mock-infected or infected with either WR, MVA or MVA Δ E3L at 10 plaque forming units (PFU)/cell, unless otherwise indicated. During this adsorption phase, cellular monolayers and viruses were incubated in EMEM containing 1% FCS. After a 1 h incubation at 37 °C, virus-containing medium was removed, and infected-cell monolayers were incubated in fresh, complete medium. In experiments where the activated form of ERK2 was detected, cells were instead incubated in medium containing 1% FCS for 24 h before infection, during the adsorption stage of infection, and for the duration of infection. Infections proceeded for indicated amounts of time, and cytoplasmic and nuclear proteins were extracted as described below.

To allow for virus attachment to, but prevent entry into, host cells, a previously described protocol was followed (Ramsey-Ewing and Moss, 1998). Briefly, the 1 h virus adsorption (MOI = 10) occurred at 4 °C, and cells were then incubated in pre-chilled medium containing anti-L1R antibodies (1:5000 dilution; a gift from Dr. Jay Hooper, United States Army Medical Research Institute for Infectious Diseases) or antibodies not reactive to the L1 protein (murine IgG; 1:5000 dilution; Sigma Aldrich). After an additional 15 min incubation at 4 °C, cells were incubated in prewarmed medium at 37 °C for 4 h. As an alternative approach to prevent virus endocytosis, cells were instead incubated in 1% FCS EMEM containing dynasore (40 mM or 80 mM; Sigma Aldrich) for 30 min prior to infection. The drug remained present in medium during the adsorption phase of virus infection (MOI = 10) and for the duration of infection. Cytoplasmic and nuclear proteins were extracted as described below.

To decrease viral gene expression, MVA particles were incubated in PBS containing 10 μ g psoralen/ml (Sigma Aldrich) and subsequently exposed to ultraviolet (UV) radiation for 5 min. These conditions, according to a previous report, would greatly inhibit viral gene expression (Tsung et al., 1996). Next, treated virions were incubated with cellular monolayers for 1 h at 37 °C at an MOI of 10. Virus-containing medium was removed and replaced with EMEM. An alternative approach to inhibit viral protein expression was to incubate cells with cordycepin (40 μ g/ml; Sigma Aldrich) for 30 min before infection. This drug was also present during the adsorption

stage of infection and for the duration of the infection. Cytoplasmic and nuclear proteins were extracted as described below.

To inhibit intermediate and late gene expression, cells were incubated in medium containing 40 $\mu\text{g}/\text{ml}$ cytosine arabinoside (Ara C; Sigma Aldrich) for 30 min. The drug remained present in medium during the adsorption phase of virus infection, in which cells were infected with 10 PFU/cell, and for the duration of infection. Cytoplasmic and nuclear proteins were extracted as described below.

Immunoblotting of cytoplasmic-extracted proteins

For all experiments, infected cellular monolayers were dislodged by scraping. Next, cells were collected by centrifugation (30 s at 10,000 $\times g$). Supernatants were removed, and cells were lysed, and cytoplasmic proteins were collected as described previously (Gedey et al., 2006). The protein concentration of each sample was determined by using the BCA assay (Pierce). An equivalent amount of protein (20 μg) from each sample was loaded into separate wells of SDS-12% polyacrylamide gels.

Proteins were separated electrophoretically and then transferred to a polyvinylidene difluoride membrane (PVDF; Millipore). Membranes were incubated in blocking buffer (TBS containing 5% milk and 0.1% Tween-20) for at least one hour. Next, membranes were incubated at 4 °C overnight in TBS-T (TBS containing 0.1% Tween-20 and 0.5% milk) containing the primary antibody below. After washing membranes in TBS-T to remove unbound primary antibodies, blots were incubated with secondary antibody consisting of either horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:10,000; Fisher Scientific) or HRP-conjugated goat anti-mouse IgG (1:5000; Fisher Scientific). The antigen-antibody reactions on each immunoblot (IB) were detected by using chemiluminescence and autoradiography. For all IBs, the Pierce Supersignal West Pico substrate was utilized, according to the manufacturer's directions. Primary antibodies included: rabbit anti-phospho-ERK1/2 antiserum (1:1000; Santa Cruz Biotechnology), which interacts with the phosphorylated form of ERK2 and the highly similar ERK1 protein; rabbit anti-ERK1/2 antiserum (1:1000; Santa Cruz Biotechnology), which recognizes the ERK2 and highly similar ERK1 protein; rabbit anti-vaccinia antiserum (1:1000; Accurate Antibody), which was raised against vaccinia virus strain Lister particles, and contains polyclonal antiserum recognizing vaccinia proteins that are present in a mature virions; rabbit anti-actin antiserum (1:1000, Fisher Scientific); mouse anti-E3L antiserum (Weaver et al., 2007) (1:1000), a gift from Dr. Stuart Isaacs (University of Pennsylvania).

For quantification of the phospho-ERK2 signals, X-ray films were digitized and analyzed by using densitometry, using the Scion Image software (Scion Corporation). The signals were quantified by using the "analyzing one-dimensional electrophoresis gels" program of Scion Image, using the "GelPlot2" macro. This method was used to compare the ratio of the band density for phospho-ERK2 only within each film image, where the film exposure time to the immunoblot was identical. A value for the phospho-ERK2-specific signal was computed by dividing the value of signal obtained from lanes containing virus-infected cellular lysate by the value of signal from lanes containing lysates of mock-infected cells. In this fashion, the value obtained for mock-infected cells was equal to one, while values of signals from MVA-infected cells were expressed as a fold-increase of phospho-ERK2 over uninfected cells.

Gel electromobility shift assays (EMSAs) of nuclear-extracted proteins

The method for isolating nuclear proteins from virus-infected cells was described previously (Gedey et al., 2006). The concentration of protein in each sample was determined by using the BCA Protein Assay Kit (Pierce). Five micrograms of each extract was incubated with 0.35 pmol of ^{32}P -labeled double-stranded oligonucleotides containing

binding sites for the NF- κB transcription factor (Promega) in Gel Shift Assay System Binding buffer (Promega) as per manufacturer's directions. Some reactions also included excess molar amounts of non-radiolabeled oligonucleotides that either contained or lacked NF- κB binding sites, or 1 μg monoclonal anti-p65 antiserum (Santa Cruz Biotechnology). Following incubation, reactions were resolved electrophoretically in a 6% acrylamide gel (Invitrogen) under non-denaturing conditions, using TBE buffer. Afterward, the gels were dried onto filter paper, exposed to a phosphorimager plate (Molecular Devices), and images were developed and analyzed using the ImageGauge and ImageReader programs, respectively (Fuji).

Luciferase reporter assay

A luciferase reporter assay was utilized to quantitate NF- κB activation in cells that were infected with poxviruses, as previously described (Shisler and Jin, 2004). For these assays, 50 ng pRL-null (Promega) and 450 ng pNF- κB Luc (Stratagene) were transfected into subconfluent 293T cell monolayers in 12-well plates by using FuGene 6 transfection reagent (Roche) according to the manufacturer's protocol. At 24 h post-transfection, 293T cell monolayers were either mock-infected or infected with MVA (MOI = 10). Alternatively, cells were infected with MVA virions pre-treated with psoralen and UV, as described above. To inhibit intermediate and late viral gene expression, infections instead occurred in the presence of Ara C, as described above. In a separate experiment to detect the effect of Toll-like receptor signaling on MVA-induced NF- κB activation, cells were co-transfected 50 ng pRL-null, 450 ng pNF- κB Luc, and 1000 ng either pERK2DN (Gedey et al., 2006) or pMyD88DN (Nichols and Shisler, 2006). At 24 h post-transfection, cells were infected with MVA (MOI = 10) or incubated in medium containing either 10 or 20 ng IL-1 β /ml medium (Sigma). Regardless of these variations, all cells were harvested 4 h post-infection by scraping, collected by centrifugation (30 s at 14,000 $\times g$), and resuspended in passive lysis buffer (Promega).

For all luciferase assays, the resultant lysates were assessed for luciferase activities as described previously (Shisler and Jin, 2004). All assays were performed in triplicate. For each experimental point, firefly luciferase activity was divided by sea pansy luciferase activity to correct for differences in transfection efficiencies. Resultant ratios were averaged and normalized to the appropriate control cells (mock-infected cells or cells co-transfected with pcDNA3.1, pNF- κB Luc and pRL-null). The value for the control cells was taken as 1. Results were displayed as relative fold change in luciferase activity as compared to either mock-infected cells or pcDNA3.1-transfected cells. Statistical significance was determined by using the Student's *t*-test.

Acknowledgments

We thank Drs. Gail Scherba and Amy MacNeill for critical review of the manuscript. We thank Drs. Jay Hooper, Stuart Isaacs and Gerd Sutter for generosity with reagents. This work was funded by NIH grant number AI055530 (JLS). SM was supported by NIH Cellular and Molecular Biology Training Grant T32 GM007283.

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